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Measuring physiological stress in the common marmoset (*Callithrix jacchus*): Validation of a salivary cortisol collection and assay technique

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ABSTRACT

Cortisol levels are often used as a physiological measure of the stress response in captive primates, with non-invasive measures of this being an important step in welfare assessment. We report a method of collecting saliva samples voluntarily from unrestrained captive common marmosets (*Callithrix jacchus*), and validate an enzyme-linked immunosorbent assay (ELISA) technique previously unused in this species. Saliva samples were collected from marmosets housed in pairs in a UK laboratory. The assay showed parallelism, precision, accuracy and sensitivity, meeting the criteria typically used to investigate the effectiveness of new analytical techniques. Use of Salimetrics® Oral Swabs considerably increased the amount of cortisol recovered in comparison with previous studies using cotton buds. However, while use of banana on the swabs can encourage chewing, it may influence results. Although increases in cortisol levels have traditionally been interpreted as an indicator of stress in primates, there are many factors that affect the hypothalamic-pituitary-adrenal axis, with some studies showing decreases in cortisol levels post-stressor. Following a likely stressful event (capture for weighing), we also found cortisol levels significantly decreased, possibly due to social buffering or 'blunting' of the HPA axis. Order of weighing also had an effect. The method therefore provided an effective non-invasive means of assessing acute changes in cortisol level that may be more useful than previous methods, improving our ability to study physiological aspects of welfare in primates. We discuss methodological considerations, as well as implications of using cortisol as a measure of stress.

1. Introduction

1.1. Cortisol as a measure of stress

When aroused, the body undergoes a set of characteristic changes, including activation of the hypothalamic-pituitary-adrenal (HPA) axis. During activation, the hypothalamus releases CRH (corticotropin releasing hormone), causing the pituitary gland to release ACTH (adrenocorticotropic hormone) into the blood, which in turn causes the adrenal gland to increase the output of glucocorticoids [64], making more energy available for immediate use and preparing the body for increased demands. While HPA axis activation is an adaptive response, very strong or prolonged periods of activation can lead to failure to reproduce [59]; abnormal behaviour [25]; impaired cognitive function [74]; immunosuppression [46], which could increase severity of infections (reviewed in [48]); or heightened risk of cardiovascular and metabolic syndromes (reviewed in [80]), all of which can have substantial implications for the wellbeing of animals.

Cortisol is the main glucocorticoid in many mammals. Numerous studies have therefore used it as an indicator of stress ([47], e.g. *Equus caballus*: [52]; *Canis familiaris*: [33]; *Macaca mulatta*: [12,56]; *Callithrix* sp.: [14,50,68]). Baseline samples can be taken, to look at relative stressfulness of certain situations, or a stressor can be imposed to examine HPA axis activation [51]. In this case, the intensity of the response from baseline to post-exposure is thought to reflect the degree of averseness, with large changes in cortisol indicating unusually high activation of the stress response, and so greater psychological and physiological stress [25]. Primates face a number of potentially stressful experiences when kept in laboratories, resulting from the captive environment and routine husbandry procedures, as well as experimental manipulations [5]. Increased cortisol levels have been well documented in primates following stressors such as loud unfamiliar noise and human activity (*Callithrix jacchus:* [14,38]), restraint (*M. mulatta*: [57]), human handling (*Saimiri sciureus*: [34]) and maternal separation (reviewed in

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[32]). Relocation (reviewed in [51]), watching other animals undergo procedures (*M. fascicularis:* [22]), isolation (*C. jacchus:* [14]), and death of a social group member (*C. jacchus*: [38]) have also been shown to be physiologically stressful.

However, the use of cortisol does have its difficulties. Levels vary across the day and season, depend on the history of the individual, the type of stressor, the presence of social companions and the collection method used [14,39,55,56,69]. For example, Johnson et al. [37] provided comprehensive data on blood cortisol levels in *C. jacchus*, measuring differences depending on sex, social status, housing and time of day, with concentrations ranging more than ten-fold from 31.2 ± 2.8 μg/dl to 317.5 ± 82.2 μg/dl. In the same species, Dettling et al. [21] found that brief separations from the family in the first month of life led to lower basal cortisol levels in 28 day old infants, compared to controls. However, there are no established normal adaptive fluctuations in levels of cortisol [25].

As well as this, there are a number of studies showing decreases in cortisol concentration following potential stressors in common marmosets. For example, Bowell [6] found that salivary cortisol level decreased significantly from baseline levels by 30 min after capture for weighing. Similarly, Cross and Rogers [15] found a consistent decrease in salivary cortisol level in all marmosets after presentation of a snake-model stimulus, although their behaviour indicated this was a clear stressor for them. Why there are such differences in findings is not immediately clear, and demonstrates the complexity of using cortisol as a measure of stress. These studies highlight the importance of collecting contextual and behavioural data to assist with the interpretation of cortisol measurements.

1.2. Collecting and measuring cortisol

Cortisol can be collected from several different mediums, giving researchers options for how to measure the physiological stress response [51]. Blood samples have traditionally been taken, often to determine acute reactions to stressors such as social separation (e.g. [36]). However, this method is often confounded by the stress of restraint or sedation. Urine can instead be collected, which is not influenced by unplanned stressful events occurring shortly beforehand. However, individual differences in output, and the precise time lag for excreted cortisol to reach the urine, can make interpretation difficult [51]. Furthermore, if 24 h sampling is required, animals have to be individually housed [67], which may confound the measurement, although primates have been trained using positive reinforcement to provide a urine sample on request (e.g. *C. jacchus*: [49]). Faecal cortisol can also be sampled [60], although like urine, lag time means pinpointing changes in relation to a specific stressor under study are imprecise, and levels depend on species, food availability and circadian variation [70,78]. To examine chronic HPA axis activity, hair has been analysed in a variety of species, with significant relationships being found between hair cortisol and stressors or abnormal behaviour (e.g. [9,17,19,20,23,24,79]). Levels of cortisol in hair are not affected by time of day or associated restraint or isolation stress, although it can be difficult to measure the time frame represented and as it is a relatively new technique, there are potential issues in how to best process the hair, extract cortisol and measure it [51].

Saliva sampling is the preferred means for assessing HPA function. Salivary cortisol is thought to reflect the non-protein bound 'free' cortisol, which is the biologically active fraction of the hormone. It is highly correlated with plasma cortisol levels (*M. mulatta*: [16]), with concentrations beginning to change within 1 min of a bolus injection of cortisol [43], indicating almost no lag time. Saliva can therefore provide a reflection of acute changes in hormone level (*M. mulatta*: [35]), which could not be investigated using metabolites within excreta, and does not cause stress like restraint or isolation, as animals can learn to chew voluntarily on collection devices without structured training (e.g. *C. jacchus:* [14]; *M. mulatta*: [45]). Previous studies have shown that coating a cotton bud in fruit is an effective method for saliva collection in the marmoset. Banana is the preferred flavour, reliably encouraging chewing, and variations in banana concentration are likely to have minimal effects on the assayed cortisol concentration [14]. Samples can be obtained quickly and in a number of different settings, while animals remain in their social group. There has therefore been significant progress in non-invasive physiological welfare assessment using hormones in saliva [35].

Once samples are collected, the enzyme-linked immunosorbent assay (ELISA) can be used to quantify the cortisol response. Saliva assays are being increasingly used to measure cortisol levels, and have been validated in a number of primate species, including baboons (*Papio h. hamadryas*: [53]), macaques (*M. mulatta*: [45]) and marmosets (*C. jacchus*: [14]). Validation involves the assessment of four established criteria, specificity, accuracy, precision and sensitivity (see [54]), to ensure the reliability of the assay and the absence of any species-specific problems. Biological relevance of the results should also be examined. However, cortisol concentrations have differed between studies (e.g. *C. jacchus*: [6,14]), which may be due to methodological differences, including the collection and assay techniques used [61,62]. Improvement and validation of methods are therefore needed, to promote more widespread use of non-invasive cortisol sampling techniques [53].

The aim of this study was to assess methods of collecting and analysing salivary cortisol samples in captive common marmosets. We explore how the use of different collection devices (cotton buds and Salimetrics® Oral Swabs, with and without banana coating) can affect results. We also validate the use of a commercially available enzyme-linked immunosorbent assay (Salimetrics®), previously unused in this species, by assessing four typically used criteria, as well as looking at changes in cortisol level following the mild routine stressor of capture and weighing, which involved short separations from the pair-mate. As direction is difficult to predict based on previous research (e.g. increases post stressor: [14]; decreases post stressor: [15]), we hypothesise that cortisol concentration will change significantly from baseline levels following brief isolation and weighing. Those weighed last in the room may also have higher cortisol levels than those weighed first. Once validated, the method can be used to monitor stress levels of marmosets in the colony, in combination with behavioural observations, and the commercial availability of the assay will encourage uptake by other facilities, increasing valid comparisons across studies.

2. Method

2.1. Animals and housing

Twenty-six adult common marmosets, housed in vasectomised male mixed-sex pairs in 3 rooms at Dstl, Porton Down, UK were studied (aged between 1 year 7 months and 2 years 7 months). All animals were purpose bred in captivity: 19 were family-reared, 7 received supplementary feeding from carestaff as infants, but remained with the family for the majority of time. All marmosets were socialised to humans from birth, with regular hand-feeding and positive interactions.

Marmosets were housed in cages measuring 100 cm wide × 60 cm deep × 180 cm high, lined with wood chippings and furnished with a nestbox, wooden platforms, perches, ropes, suspended toys and a wire veranda. All marmosets had ad libitum access to water, and food was delivered twice a day. Primate pellets (40/pair) were fed in the morning, and a variety of fruit (1 piece/animal) was provided in the afternoon. This was supplemented with malt loaf, egg, rusk, mealworms, dates, peanuts and bread on alternating days. Gum arabic and milkshake (with added Vitamin D once a week) were also given twice a week, and a constant supply of forage mix was available. Enrichment was introduced once a week, where paper parcels, cardboard boxes or bottles were provided, with forage mixed into sawdust. Temperature and humidity were at 23-24 °C and 55 ± 10% respectively. Lighting